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Just in case it rains: building a hydrophobic biofilm the *Bacillus subtilis* way

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Over the millennia, diverse species of bacteria have evolved multiple independent mechanisms to structure sessile biofilm communities that confer protection and stability to the inhabitants. The Gram-positive soil bacterium *Bacillus subtilis* biofilm presents as an architecturally complex, highly hydrophobic community that resists wetting by water, solvents, and biocides. This remarkable property is conferred by a small secreted protein called BslA, which self-assembles into an organized lattice at an interface. In the biofilm, production of BslA is tightly regulated and the resultant protein is secreted into the extracellular environment where it forms a very effective communal barrier allowing the resident *B. subtilis* cells to shelter under the protection of a protein raincoat.

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Introduction

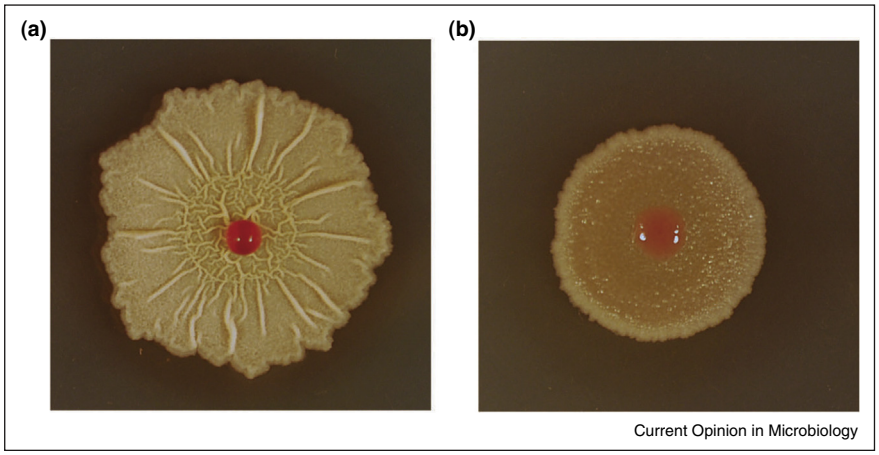
Biofilms are social microbial communities that have garnered attention due to their remarkable resistance towards antimicrobials and sanitizers by comparison with their free-living bacterial counterparts [1]. These properties render biofilms both useful, since they can be used as removal agents of environmental waste [2] and harmful, as they can accumulate in industrial and hospital settings causing biofouling problems [3,4] and chronic infections [5], respectively. The hallmark of biofilm formation is the production of the extracellular matrix that holds the microbial multilayers together and provides a favourable environment for biofilm stability [6]. The most predominant components of the biofilm matrix, across a variety of microorganisms, are exopolysaccharides, extracellular DNA and secreted proteins, many of which are fibre forming [7]. The properties imparted by these matrix molecules influence the overall features of the mature biofilm [8–11].

The surface of the biofilm formed by the Gram-positive soil bacterium *Bacillus subtilis* is exceptional: it exhibits resistance towards gas penetration and is highly hydrophobic, capable of preventing water, solvents, and commercial biocides from reaching the core of the community [12••] (Figure 1a). These findings prompted studies to unravel the mechanisms that allow *B. subtilis* to exhibit these features and it was uncovered that a small protein called BslA (for Biofilm Surface Layer protein A) was directly linked [9••] (Figure 1b). In this review we will briefly outline the regulatory network controlling BslA production, and then detail current knowledge of the function, structure and mode of action of this surface active protein.

Controlling the production of BslA

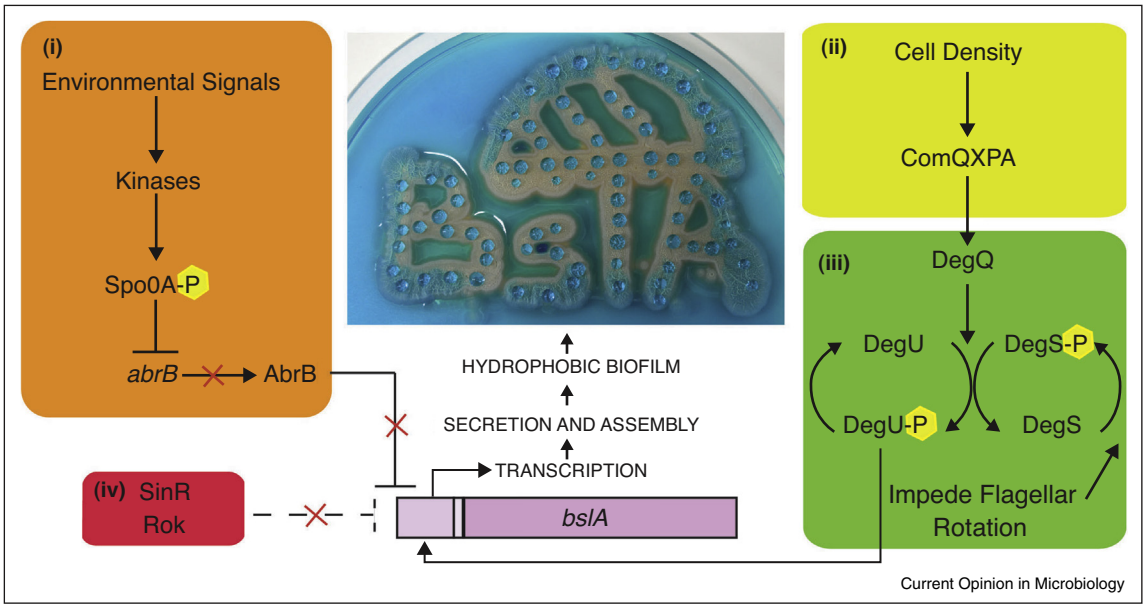
The transition from planktonic cells to a sessile life is an elaborate process involving gene regulation and the production of large macromolecules. The extracellular matrix of the *B. subtilis* biofilm comprises a large exopolysaccharide [13•], which is synthesized by the products of the *epsA-O* operon, TasA protein fibres, which are synthesized by the *tapA-sipW-tasA* operon [14,15] and the small secreted protein BslA, the focus of this review. Transcription of *bslA* is tightly regulated by a suite of repressors and activators which form a regulatory network (Figure 2). AbrB is a pleiotropic regulator [16,17] which binds directly to the *bslA* promoter region and inhibits transcription [18]. In response to environmental signals, which include nutrient availability [19] and the extracellular lipopeptide surfactin [20], the regulator Spo0A becomes phosphorylated *via* a phosphorelay [21] and subsequently directly represses transcription of *abrB* [22], thus allowing production of BslA [18] (Figure 2; module (i)). Additionally, when cell density increases, the ComQXPA two-component signal transduction system induces the transcription of *degQ* [23] (Figure 2; module (ii)). The small DegQ protein aids the phosphorylation of DegU (hereafter DegU∼P) by enhancing transfer of the phosphoryl moiety from DegS to DegU [24•]. Low levels of DegU∼P stimulate transcription of several genes needed for biofilm formation, including *bslA* [24•,25] (Figure 2; module (iii)). In fact, replacing the native *bslA* promoter region with a heterologous (IPTG inducible) promoter can compensate for the absence of

Figure 1



The biofilm formed by *Bacillus subtilis* strain NCIB3610 manifests as an architecturally complex, hydrophobic, community on MSgg agar [13•]. The wild-type **(a)** and the *bslA* deletion strain **(b)** (NRS2097 from [18]) were grown for 48 h on MSgg agar prior to photography and placement of a 5 μl droplet of coloured water on the upper surface of the biofilm.

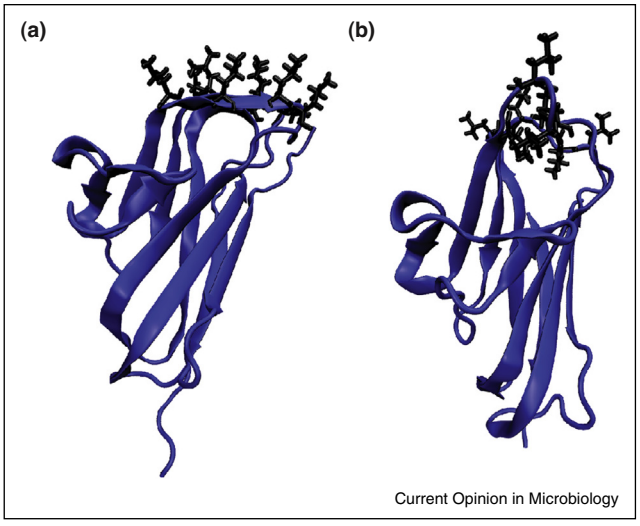
Figure 2



The regulatory pathway controlling *bslA* transcription consists of four modules: (i) repression is directly mediated by AbrB and released upon activation of Spo0A (by phosphorylation) which lowers the level of AbrB; (ii) Generation of DegQ by the ComQXPA regulatory system is activated at high cell density; (iii) activation of *bslA* transcription by phosphorylated DegU is a process that is activated upon impedance of flagellar rotation and aided by DegQ; and (iv) indirect repression of *bslA* mRNA production by SinR and Rok. The arrows represent activation and the T-bars inhibition; the red crosses represent pathways that are inactive under biofilm forming condition; and the 'P' in the yellow shape represents phosphorylation.

degU, demonstrating that the biofilm defect displayed by the *degU* mutant is due to the lack of BslA [26]. The exact environmental signals that trigger activation of DegS are unknown but impedance of flagellar rotation is linked with an increase in *bslA* transcription [27]. Deletion of *sinR*, which encodes the master repressor of *eps* and *tapA-sipW-tasA* operons [28], also leads to enhanced transcription of *bslA* [18]. While a direct interaction of SinR with the promoter region of *bslA* was not detected, increased synthesis of the exopolysaccharide triggers transcription of *bslA* in the *sinR* mutant [18]. Given the explicit role for SinR in controlling the production of the

Figure 3



The atomic structure of BslA (covering amino acids 48-172; PDB4BHU) in the hydrophobic cap 'OUT' (chain C in the decameric crystal structure) **(a)** and hydrophobic cap 'IN' (chain I in the decameric crystal structure) **(b)** forms. The side chains of the leucine and isoleucine amino acids in the hydrophobic cap are presented.

exopolysaccharide and the TasA-fibres [29], these data suggest that *B. subtilis* has a mechanism of detecting and responding to the local environment allowing coordinated production of the matrix molecules. Finally, another transcription regulator, namely Rok, indirectly regulates *bslA* transcription [30], but how Rok integrates into the overarching regulatory network is currently unknown (Figure 2; module (iv)).

The structure and biological function of BslA

At the sequence level BslA is an unassuming ~19.2 kDa protein with its only defining feature being a canonical Sec-dependent signal sequence [26]. Structurally BslA resembles members of the immunoglobulin superfamily [31••], comprising one 3_{10} helix and 13 β-strands that form two distinct opposing faces of the molecule (Figure 3). Significantly, the Ig scaffold is appended with a three-stranded β-sheet cap consisting of hydrophobic amino acids that form a large surface-exposed patch (Figure 3a). Based on the distinct physical properties of its surface, BslA has been characterized as a nanoscale biological Janus ellipsoid [32]. The *bslA* mutant is unable to form spore containing, architecturally complex biofilms that are archetypal of the wild isolates of *B. subtilis*, moreover the structures formed have a striking wetting phenotype (Figure 1b) [9••,26]. The loss of hydrophobicity was revealed using a simple assay where droplets of water are placed on the upper surface of the biofilm. For the wild-type biofilm, the water retains a spherical shape (Figure 1a), whereas the droplet instantly disperses on the surface of the *bslA* biofilm (Figure 1b) [9••].

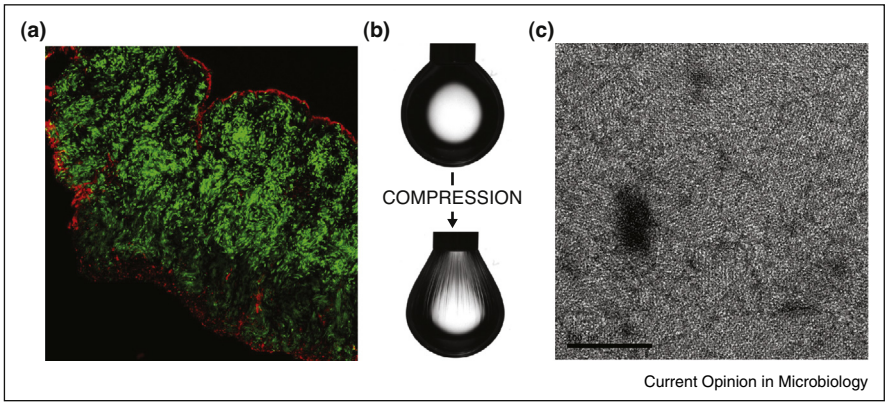
BslA is a secreted protein [26], and consistent with functionality in the extracellular environment, addition of recombinant fluorescently labelled (using DyLght594) BslA (comprising amino acids 42-181), can reinstate biofilm formation and hydrophobicity to the *bslA* mutant. Microscopy analysis reveals that the protein provided exogenously forms an isolated layer on the upper surface of the community [9••]. The discrete localization profile of BslA was confirmed in the wild-type biofilm by immunofluorescence coupled with confocal microscopy of thin biofilm cross-sections, where a distinct layer of staining, specific to production of BslA, was observed at the periphery of the biofilm [31••] (Figure 4a). The BslA layer also plays a role in the overall physicomechanical properties of the biofilm and influences surface stiffness and roughness, as determined by atomic force microscopy [33]. Generation of a suite of site-directed mutants, combined with both *in vivo* and *in vitro* analyses, substantiated the importance of the hydrophobic cap for forming architecturally complex biofilms and also conferring water resistance to the biofilm [31••]. It is highly likely that localization of BslA depends on interaction with the biofilm exopolysaccharide, as deletion of the *eps* gene cluster renders the biofilm entirely wetting [9••,12••] and also allows release of BslA into the culture supernatant [9••]. How this occurs, and the specific interactions involved, remains to be delineated.

Formation of an organized protein layer

Analysis of recombinant BslA revealed self-assembly capabilities [9••,31••]. At a liquid-oil interface this manifests as formation of a stable film that can resist compression (Figure 4b) [31••]. Consistent with the hydrophobic cap engaging with the interface, mutation of the central leucine residues in the cap region weakens the interaction with the oil interface and upon compression the protein film rapidly relaxes; the *in vivo* consequence is a fully wetting biofilm [31••]. Transmission electron microscopy analysis revealed that BslA forms a well-ordered 2D rectangular lattice at the air-water interface [34••] (Figure 4c). Surface compression isotherm and atomic force microscopy showed that the monolayer of BslA forms a solid, tightly packed membrane-like structure [35]. Furthermore, vibrational sum frequency generation spectroscopy uncovered a sharp amide I vibrational band in the spectrum at the air-water interface, reflecting the well-ordered film formed by BslA [35].

The surface exposure of a hydrophobic patch, and the ability to form a stable elastic film, alongside its function within the bacterial community, are features that are reminiscent of fungal hydrophobins, which form a proteinaceous hydrophobic coat on the surface of the fungal hyphae [36].

Figure 4



(a) BslA forms a layer around the *B. subtilis* community as determined using immunofluorescence coupled with confocal microscopy. Shown is a cross section of the biofilm where the cells are shown in green and BslA in red (visualized by immunofluorescence analysis). The image is taken with permission from [31••]; **(b)** Recombinant BslA forms a film at the oil-water interface which develops stable wrinkles upon compression. The image is taken from with permission [21] where a droplet of BslA at a low concentration in phosphate buffer was expelled into an oil bath contained in a square cuvette. The oil phase is white (the boundary of the cuvette is not visible) and the protein solution is grey. An initial droplet of 40 μl was expelled and then 5 μl was retracted allowing the wrinkled protein layer at the oil–water interface to be visualized; **(c)** BslA forms an organized rectangular lattice as visualized by transmission electron microscopy reproduced with permission from [34••] where the scale bar is 100 nm.

Therefore, despite the lack of sequence homology with hydrophobins, and the distinct structural configuration, this led to the designation of BslA as a 'bacterial hydrophobin'. From a biotechnology point of view the unique characteristics of this protein render it an outstanding candidate for a number of applications in various fields, such as the synthesis of new surface-active biomimetic materials [37], or as an ingredient in formulations that contain multiple different phases such as ice cream [38].

BslA engages with the interface using limited structural rearrangement

Detailed biophysical and computational analysis of BslA revealed a 'smart' mechanism by which BslA remains soluble within the aqueous biofilm environment despite the presence of a large hydrophobic cap, assembling into a protective surface layer only when it reaches an interface. In aqueous solution, the cap region of monomeric BslA is unstructured, where the side chains of the hydrophobic residues that constitute the cap are facing towards the interior of the protein (Figure 3b) [34••]. When BslA reaches the air-water interface, the hydrophobic cap changes conformation such that the side chains re-orientate towards the air and form the three-stranded β-sheet cap, a transition that gives rise to a small energy barrier to adsorption. These two structural conformations were serendipitously found in the decameric crystal structure of BslA in different local solvent environments [34••]. The fact that the cap region undergoes the conformational change only after adsorption presumably allows the protein to avoid undesirable interactions within the biofilm and to reach the interface. This is the first time such a stabilizing mechanism has been reported.

Despite the detailed mechanistic understanding of BslA function, outstanding questions still remain. For example, the amino acids that mediate the lateral protein-protein interactions that give rise to the ordered 2D lattice remain unidentified, although it is possible that the restructured cap gives rise to an extensive β-sheet array. The fact that the protein is stable and monomeric in aqueous solution when in a reducing environment that prevents disulphide bond formation [34••] provides additional evidence for self-assembly being linked to this restructuring, ensuring the protein self-assembles only in the right place. Additionally, the precise mechanism that triggers the structural rearrangement of the cap remains unidentified. It is possible that the protein kinetically interconverts between the two forms; however, it is difficult to see how this would prevent undesirable interactions with components deep within the biofilm.

Forming a bacterial raincoat

B. subtilis is commonly found in the upper layers of the soil [39] where it accumulates in dense communities, forming biofilms that are beneficial for plant growth and protection from pathogens. The *B. subtilis* biofilms form a mutualistic interaction with plants rhizome systems, providing preemptive colonization, which prevents other pathogens from infecting the plant [40] whilst the bacteria benefit from the nutrients released by the root system. Taking into account the natural habitat of *B. subtilis*, one could hypothesize that the water repellency would give the bacterium an evolutionary advantage. It might enhance colonization of the plant root, it may allow the bacterial community to be more resilient

towards adverse weather phenomena, such as rain, or it could shield the community from antimicrobial agents produced by local competitors. In short, the remarkable ability of BslA to form a robust, highly-ordered and stable film located at the water-air interface, allows it to encase the entire sessile community in a protective raincoat. This raises the interesting question, however, of how a water-repellent community takes in nutrients from the surrounding environment. It has been shown that the wrinkled biofilm surface forms an interconnected network of liquid channels [41] (see Figure 2), however, it is difficult to envision how the demonstrated presence of BslA at the periphery of the biofilm facilitates nutrient flow through these channels and this remains an open question.

Outlook: Looking beyond *Bacillus subtilis*

Currently few bacterial species have been reported to exhibit hydrophobic properties. For the *Vibrio cholerae* mature biofilm, Bap1 is a 26.3 kDa proteinaceous matrix component, which is produced during the latter stages of biofilm formation [42], and greatly influences *V. cholerae* hydrophobicity [43]. The *Δbap1* deletion strain forms pellicles which are unable to remain at the surface upon transfer from the growth medium into deionized water. This is in contrast to pellicles formed by wild-type *V. cholerae* which spread after contact with the air–water interface and reinstate their previous form [43]. How Bap1 mediates buoyancy in the pellicle and contributes to hydrophobicity is unknown, but real-time confocal microscopy analysis of developing biofilms demonstrates that it coats the substratum at the base of the biofilm, extending away from the cell biomass [44], which is reminiscent of the protein layers formed by BslA [9••,31••]. In *Pseudomonas* sp. UK4, overexpression of the *fap* operon, which is responsible for the expression of amyloid-like fibrous proteins found in the extracellular matrix of biofilms [45], led to increased hydrophobicity alongside a 20-fold increase in biofilm stiffness [46]. As a final example, *P. putida* was found to release membrane vesicles as a response to different stress conditions, which increased cell surface hydrophobicity and triggered biofilm formation [47]. Each of these methods is evolutionarily distinct from the BslA system used by *B. subtilis*, thereby suggesting that there is general advantage for bacteria to be able to control the hydrophobicity of their community. The development of novel bioinformatics methods or utilization of systematic screens, for the identification of new proteins involved in bacterial hydrophobicity will help reveal the scale, scope, mechanism, and function of these fascinating systems.

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